Using CRISPR-Cas9 HDR positive controls

Alt-R CRISPR-Cas9 HDR positive controls are experimentally validated controls that consist of both a guide RNA (available as the 2-part crRNA:tracrRNA or the single guide sgRNA) and a single-stranded HDR donor template.

The provided guides target either the *AAVS1* (Human) or *Rosa26* (Mouse) safe-harbor locus to create a CRISPR-Cas9–mediated double-stranded break. The provided HDR donor templates will introduce a six-base insertion (EcoRI restriction enzyme recognition sequence) at the CRISPR-Cas9 cleavage site in cells that have undergone HDR. The guide and HDR donor template sequences for both human- and mouse-specific designs are shown in Table 1 with the EcoRI insertion sequence indicated in red.

Table 1. Positive control guide RNA and HDR donor templates.*

Species	Guide (spacer sequence)	HDR donor†	Edit location
Human (*Homo sapiens*)	CCTCTAAGGTTTGCTTACGA	AGCCATCTCTCTCCTTGCCAGAACCTCTAAGGTTTGCTTA**GAATTC**CGATGGAGCCAGAGAGGATCCTGGGAGGGAGAGCTTGGCA	chr19:55115594
Mouse (*Mus musculus*)	TAACAACTCAGAGCGACTTT	CTCCACTGCAGCTCCCTTACTGATAACAACTCAGAGCGAC**GAATTC**TTTGGGAGAGCAAGTGCTTCCTGCCTCCAAAACAGCCCAA	chr6:113076232

* Positive controls (guide RNA and HDR donor template) are available at www.idtdna.com/HDRDonorOligos.

† GAATTC = EcoRI recognition site

Verification of insertion

CRISPR genome editing takes place within 48–72 hr in the cell lines we tested. Typically, we collect genomic DNA 48 hr after the delivery of CRISPR-Cas9 reagents and assess the overall editing efficiency of both NHEJ and HDR pathways.

For the quantification of HDR events, 1 of the following 3 methods can be employed:

1. Restriction fragment length polymorphism (RFLP) assay

   Insertion of a restriction enzyme recognition sequence facilitates the subsequent detection of successful HDR using RFLP analysis. Primers should be carefully designed to prevent unwanted amplification of donor DNA, which potentially leads to false positive results. After PCR amplification, the restriction enzyme EcoRI can be used to enzymatically digest the PCR product and the frequency of insertion can be estimated by measuring the resulting fragments.

The primers shown in Table 2 can be used to amplify the AAVS1 or Rosa26 region by PCR for an RFLP assay.

Target	Primer	Sequence*	Full-length (bp)	Fragment 1 (bp)	Fragment 2 (bp)
Human *AAVS1*	Forward	GCCAAGGACTCAAACCCAGA	1037	561	476
	Reverse	CCCCGTTCTCCTGTGGATTC			
Mouse *Rosa26*	Forward	CGAGGCGGATCACAAGCAAT	571	431	140
	Reverse	GTGCAAGCACGTTTCCGACT			

* Order these primers as custom oligos (ordering page accessible from **DNA oligos** at www.idtdna.com/DNA.)

2. Sanger sequencing

   To check the DNA sequence of your edited locus at the single-nucleotide level, you can use Sanger sequencing. This is a straightforward and cost-effective method to check on-target editing at a single locus. Various software programs are available online to analyze complex Sanger sequence traces representing samples of mixed sequences after CRISPR-Cas9–mediated editing.

3. Next generation sequencing

   Next generation sequencing offers rapid and cost-effective approaches for measuring desired changes to on-target loci, as well as identifying genome-wide, off-target cleavage events that result in mutagenic repair. Ideally, to verify the absence of off-target sites, cells that undergo genome engineering should be fully characterized by NGS methods. NGS is an efficient, quantifiable, and comprehensive approach for measuring the levels of on- and off-target editing.

Functional performance of HDR positive controls

HDR rates will vary with cell type and delivery conditions. HDR rates in several commonly used cell lines are shown in Figure 1.

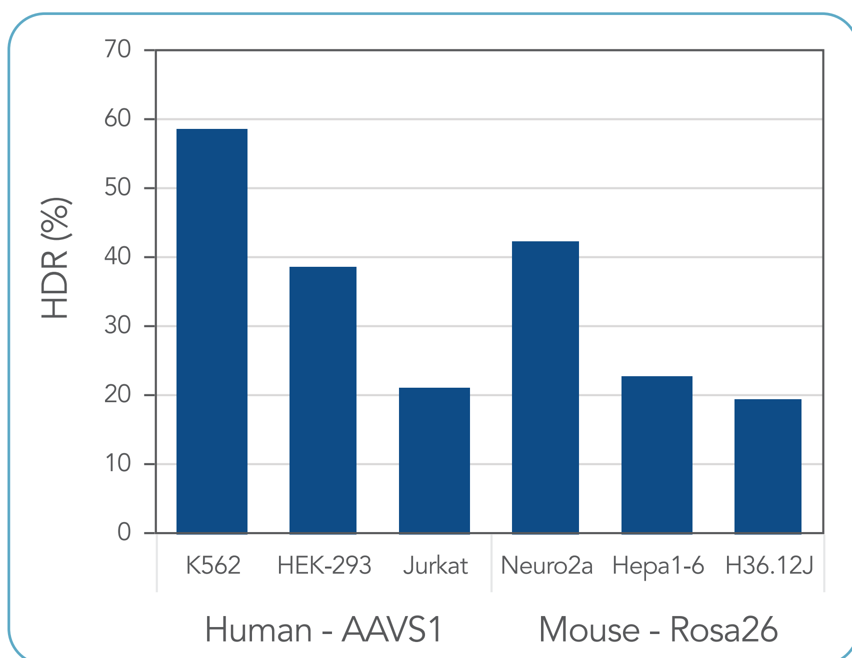

Figure 1. Representative positive control HDR rates in commonly used cell lines. RNP complexes (2 µM) consisting of Alt-R S.p. Cas9 Nuclease V3 complexed with Alt-R crRNA and tracrRNA targeting either the *AAVS1* or *Rosa26* safe-harbor locus were delivered by the 4D-Nucleofector System (Lonza). Alt-R HDR donor templates were included at 0.5 µM (K562, HEK-293, Jurkat human cells) or 3 µM (Neuro2a, Hepa1-6, H36.12J murine cells) along with 2 µM of Alt-R Cas9 Electroporation Enhancer. HDR efficiency was measured by amplicon sequencing on an Illumina® MiSeq® system (K562, HEK-293, Jurkat) or by EcoRI restriction fragment length polymorphism (RFLP) assay (Neuro2a, Hepa1-6, and H36.12J).

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